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THE LABORATORY DIAGNOSIS OF GLANDERS.*

E. Marion Wade.

(From the Bacteriological Laboratory of the Boston Board of Health.)

Glanders, although one of the earliest known diseases of horses, was first considered infectious in the 17th century, and toward the end of the 18th Abildgaard and Viborg proved it to be so. This was later disputed by various authorities, until Rayer in 1837 demonstrated its transmissibility to man and Chaveau in 1868 showed that the virus was contained in the lesions of the affected animal. Even after the infectious nature of the disease was determined, many varying opinions were held as to the causative agent, until in 1882 Loeffler and Shutz settled all disputes by finding an organism which fulfilled all of Koch's postulates. While these discoveries meant much, still the bacteriological diagnosis had been very little aided, as the isolation of the specific organism by plating from lesions or nasal discharges is a long and tedious process and is met with very little success, as will be shown later. In 1886 Strauss proposed the intraperitoneal inoculation of male guinea-pigs with the suspected material as a quick means of diagnosis. Usually within three to four days the scrotum becomes red and shining and suppuration occurs. This pus contains the specific bacillus usually in pure culture.

Mallein, a toxin of the glanders bacillus, was first discovered by Helman and Kalning in 1891. They used filtered aqueous and glycerin extracts of potato cultures. Roux cultivated *B. mallei* in flasks of five per cent glycerin broth. This latter is the method generally used. The cultures are grown for six weeks in some laboratories, for five months in others. This is then boiled to kill the organisms and concentrated to one-half its volume, filtered through a Berkefeld candle and diluted with an equal volume of one per cent carbolic acid, making the final product 0.5 per cent carbolic acid. The diagnostic dose is 2 c.c. At the present time the ophthalmic test is being tried, using a concentrated extract,

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and is meeting with encouraging results. Although some work had been done with the agglutination tests by McFadyean and others and Pfeiler and Konew had used the precipitation test, the next marked advance in the laboratory diagnosis was made in 1910 when Schutz and Schubert applied the complement-fixation test to glanders. This test supplemented by the agglutination test is at the present time taking the leading place in the diagnosis of glanders by public health laboratories and cattle bureaus.

The two most essential factors in all laboratory diagnoses are accuracy and early reports, and a comparison of the methods in use for glanders along this line is interesting. In a series of 1,600 examinations by the use of guinea-pigs after the Strauss method covering a period of six and one-half years 497 or 31.16 per cent gave a positive result. Of these positive cases the cultures made direct from the suspected material on potato gave a characteristic growth in only 62 instances or in 12.5 per cent. The remainder of the cultures were overgrown either with mold or other organisms and any attempts to isolate *B. mallei* would have been unsuccessful. Results obtained from a small series of such attempts seem to justify this statement. In 24 of the 62 cases the inoculated guinea-pigs had shown no lesions up to the time of development of a characteristic growth on the culture and the same pigs were reinoculated with an aqueous suspension of the culture and developed typical scrotal lesions on the second or third day. In these cases therefore the culture made direct from the suspected material enabled the giving of a positive report which might otherwise have been missed. In the remaining 38 cases the pigs showed lesions at the time of the characteristic growth on potato. This leaves 435 out of the total 497 in which the culture direct was of no assistance although 14 times very suspicious organisms had been found in the smear made from the material. Before a final positive report is given by this method three things are essential—typical lesion in the scrotum of the guinea-pig, typical organisms in the smear from the pus at the time of autopsy of the pig, and a characteristic growth on potato from the lesion in the pig. In several instances a similar lesion has been produced by other organisms—*B. pyocyaneus* being the most common. This can be readily distinguished from *B. mallei* by its much darker color on potato and its more moist and abundant growth often extending around the whole surface of the medium.

Varying opinions are held as to the length of time that *B. mallei* will retain its virulence upon artificial media without passage through animals—from two or three generations to at least two months. Ten strains isolated between September 23 and October 31, 1911, have been twice passed through guinea-pigs—once November 29, 1911, and the second time March 4, 1912. Four of these cultures were inoculated September 6, 1912, after being on artificial media six months, and in each case the pig developed a typical Strauss reaction on the second day, as had been the case in the previous inoculations. From this it is evident that at least some strains of *B. mallei* retain their virulence on artificial media for six months. These same cultures will be kept and tested periodically. Much still remains to be done on the length of life of the organism in a dried condition as met with in infected stables, on the tie posts and halters, and the chances of conveying infection from horse to horse by the walls and tie chains in blacksmith shops, not to mention the public watering trough.

The diagnosis of glanders by the precipitation test recommended by Pfeiler in Germany and Konew in Russia has not met with general corroboration. In a few cases tried by the writer the results were not found to agree with the diagnoses by other methods.

The agglutination test for glanders was first suggested in 1896 by McFadyean after Widal's test had been adopted for typhoid fever. It was not extensively used, however, until Schutz and Meissner established its value in 1905. Since this time the test has been widely employed but it is not absolutely reliable as by it alone some cases would be missed. Whether or not it is more reliable in acute and less so in chronic cases has yet to be proved. The macroscopic agglutination test with the aid of the centrifuge seems to be the most reliable of the agglutination tests. To make the test fluid pure cultures of several strains of *B. mallei* are grown on two and one-half per cent glycerin agar in flasks or, as is more commonly the case, in wide-mouth bottles (the media covering one of the large surfaces), in order to get a large area for growth. After 24 to 36 hours in the incubator at 37° C. the cultures are killed by heating to 60° C. for two hours and then washed off the surface of the media with physiological salt solution containing 0.5 per cent carbolic acid. The fluid is filtered through ordinary filter paper and diluted to the proper density by comparison with a known agglutination fluid and by testing against sera of known agglutination power. The fluid should not be tested until it has stood at ice-chest temperature for eight to 10 days. It keeps well for two to three months.

In making the test a basic dilution of the serum to be examined is made and from this other dilutions can be prepared. A dilution of 1:40 has been recommended for the basic dilution but the writer has used 1:80 in order to lessen the possibility of error in the high dilutions where 0.01 c.c. would be used. Varying amounts of the basic dilution are placed in tubes according to the table below.

1:600	by using	.26	c.c. of basic dilution.
1:800	“ “	.20	“ “ “ “
1:1000	“ “	.16	“ “ “ “
1:1200	“ “	.14	“ “ “ “
1:1400	“ “	.12	“ “ “ “
1:2000	“ “	.08	“ “ “ “
1:4000	“ “	.04	“ “ “ “
1:8000	“ “	.02	“ “ “ “

To each of these tubes two cubic centimeters of agglutination fluid is added. Two control sera—a known positive and a known negative—are run with each set of tests. The tubes are placed in the incubator at 37° C. for a half hour, then centrifugalized for 10 minutes at a speed of 1,600 revolutions a minute. The readings are made after the tubes have stood at room temperature for at least two hours, and if there is no immediate hurry for a report it is better to let them stand over night. The readings can best be made by looking down through the tubes against a dark surface. In a positive reaction the bacteria will be seen in clumps and spread over the bottom of the tube, but in a negative case there will be a round, dense-white precipitation in the center of the bottom of the tube. There is so much variation in the agglutinating power of normal serum that a positive report has not been given unless an agglutination has been obtained at a dilution of 1:2000. If there has been an agglutination at 1:1400 an atypical report has been given, and other specimens of blood asked for. The dividing line between a positive and a negative report should be established by the laboratory making the tests, as different agglutination fluids made by different workers seem to differ although of the same apparent density. The fluid in use in the tests reported below has been made from eleven strains of *B. mallei* isolated in September and October, 1911.

In the tests by complement fixation the technic followed has been that recommended by Mohler and Eichhorn,[1] with a few modifications. This differs from the technic usually employed in the Wassermann test mainly in the titration of the complement and using the smallest amount which with two units of amboceptor gives complete hemolysis instead of using always a 10 per cent solution and varying the amount of amboceptor used and thus having an excess of complement.

In the preparation of the amboceptor it has been found better to lessen the time between the inoculations, thereby lessening the chance of losing the rabbit by anaphylaxis. An amboceptor titrating 1:2000 was obtained in one instance by giving inoculations of 5, 10, and 15 c.c. respectively on three successive days and bleeding on the ninth day after the last inoculation. This amboceptor retained a strength of 1:1500 until it was all used six months later. Reinoculations of 5, 10, and 15 c.c. were made into the same rabbit at three-day intervals but it died on the second day after the last inoculation although appearing to be in perfect health on the night previous.

The glanders antigen has been the one troublesome feature in the test, especially because of its instability. Also some clinical cases of glanders on which guinea-pigs have given a positive result and of sufficient duration to lead to the expectancy of a positive reaction to complement fixation have failed to give it. Most of the antigens in use at the present time in this country are shaken but in Germany the shaking is being dispensed with. Syphilitic antigen is being prepared without shaking, as is also gonococcus antigen, and many working with complement-fixation tests here are beginning to follow the example of the Germans. For this reason it has been thought advisable to try some glanders antigens in the same way. The first attempt was to grow the cultures on neutral veal agar and the growth was very scant and somewhat discouraging, but it was thought that a better growth might be obtained after several transplants had been made on this medium. The growth was very little better but an antigen was made. This was unsatisfactory. Next neutral glycerin agar was used and the growth was also scanty, but an antigen made in the same manner gave a titre of 1:10. The glanders bacillus prefers slightly acid media to grow upon so the ordinary glycerin agar one per cent acid was next used, this being the medium on which the cultures had been growing since their isolation. The antigen made in the same way from this medium yielded a titre of 1:300. The following technic was employed in making the antigen: Eleven strains of *B. mallei* were used, the growth being washed off the surface of the media with sterile distilled water to give a milky suspension. This was then autolyzed for one-half hour at 56° C., after which it was filtered through a Berkefeld candle. This filtrate was then sterilized by heating to 56° C. for two hours on three successive days. For use the antigen is diluted with nine per cent salt solution to make a product 0.9 per cent saline (0.9 c.c. antigen + 0.1 c.c. nine per cent salt solution), otherwise hemolysis might be produced. This antigen has been used in a duplicate series of 117 sera with absolutely reliable results. The ease with which the antigen is prepared and the saving of time recommend its adoption.

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[1] *Bulletin 136*, Bureau of Animal Industry, Washington, D.C.

One other modification in technic has been tried with very satisfactory results. In the second set of tubes in each test a double quantity of antigen has been used and in the control tube 0.4 c.c of serum instead of 0.2 c.c. When the control tube containing 0.4 c.c. of serum has completely hemolyzed the readings can be made, as no inhibitory action could then be due to 0.2 c.c. serum. It has been found in many instances that a much sharper reaction has been obtained by using this double quantity of antigen with the 0.2 c.c. serum and the reading changed from a ± to a + or even a ++.

During the past year 456 sera have been tested by both complement fixation and agglutination and the results have agreed in 373 instances or almost 82 per cent. The results on the remaining 84 sera from 76 cases are interesting, as will be seen from the following table.

TABLE I.

	NUMBER OF CASES	FINAL OUTCOME		
		+	Questionable	–
C.F.+ Ag. atyp.	6	6	...	...
C.F. atyp. Ag.+	10	8	...	2
C.F.– Ag.+	36	21	4	11
C.F.+ Ag.–	5	3	...	2
C.F. atyp. Ag.–	3	2	1	...
C.F.— Ag. atyp.	16	...	...	16
Total	76	40	5	31

The two cases listed as "negative" with C.F. atyp. Ag.+ are from the same stable, neither showing symptoms seven months later—the mate of one had been killed as glandered and from the other an atypical mallein reaction had been obtained. Of those with C.F.–Ag.+ in three cases the agglutination test was made after the horses had been released on the negative complement-fixation report and had changed owners, so no subsequent history has been obtainable, but the veterinarian reports that they were probably glandered horses. In the fourth case blood taken two weeks later gave a positive result by complement-fixation and also by agglutination, but the horse is still living seven months later and shows no symptoms. These four are therefore put in the questionable classification. Ten of the cases called negative were from the routine taking of specimens from stables in which glanders existed, but the horses show no symptoms to date. In the other case the horse was killed but at autopsy no glanders lesions were present—the mate of this horse was glandered.

The two negative cases with C.F.+ Ag.– were also from the routine taking of specimens from a stable and still show no symptoms. The one questionable case with C.F. atyp. Ag.– is at present under observation. Of course some of the cases called negative may have glanders in a latent form, but as no evidence of it exists at the present time it seems fair to class them as negative as horse owners object to losing horses which show absolutely no external signs and are in apparent good health. This summary of cases seems to indicate that a positive report by complement-fixation or agglutination is strong evidence of glanders while an atypical agglutination without some fixation of complement should not be so considered.

In 135 cases guinea-pig tests have been made as well as blood tests and Table 2 shows the results of these tests.

TABLE 2.

SUMMARY OF CASES ON WHICH BOTH STRAUSS AND BLOOD TESTS WERE MADE

Positive Cases		*Negative Cases*	
Both +	56	Both −	32
Blood + Strauss−	30	Blood − Strauss Uns	5
Blood + Strauss Uns	10		
Blood − Strauss+	2		37
	98		

This shows that in 45 instances out of 135 or 33.3 per cent the pigs failed to give a reliable diagnosis and two cases in which the blood test failed but in one of these instances the agglutination test was not made.

CONCLUSIONS.

1. The diagnosis of glanders by the complement-fixation test supplemented by the agglutination test on all negative sera is the quickest and most reliable test at the present time.
2. Glanders antigen prepared without shaking but filtered through a Berkefeld candle gives reliable results and yields a more stable product.
3. Normal horse serum varies widely in its agglutinating power, therefore a weak positive should be considered in conjunction with the complement-fixation test or with clinical symptoms.
4. Antigens and agglutination fluids should be prepared from several strains of *B. mallei.*
5. While a positive result from guinea-pig inoculation is conclusive evidence of the presence of glanders, failure of the pigs to develop lesions is not proof of its absence.